

# Chemotaxis Inhibitory Protein of *Staphylococcus aureus*, a Bacterial Antiinflammatory Agent

Carla J.C. de Haas,<sup>1</sup> Karin Ellen Veldkamp,<sup>1</sup> Andreas Peschel,<sup>1,2</sup>  
Floor Weerkamp,<sup>1</sup> Willem J.B. Van Wamel,<sup>1</sup> Erik C.J.M. Heezius,<sup>1</sup>  
Miriam J.J.G. Poppelier,<sup>1</sup> Kok P.M. Van Kessel,<sup>1</sup> and Jos A.G. van Strijp<sup>1</sup>

<sup>1</sup>Eijkman-Winkler Institute, University Medical Center Utrecht, 3584 CX Utrecht, Netherlands

<sup>2</sup>Cellular and Molecular Microbiology, Medical Microbiology and Hygiene Department, University of Tübingen, 72076 Tübingen, Germany

## Abstract

Leukocyte migration is a key event both in host defense against invading pathogens as well as in inflammation. Bacteria generate chemoattractants primarily by excretion (formylated peptides), complement activation (C5a), and subsequently through activation of leukocytes (e.g., leukotriene B<sub>4</sub>, platelet-activating factor, and interleukin 8). Here we describe a new protein secreted by *Staphylococcus aureus* that specifically impairs the response of neutrophils and monocytes to formylated peptides and C5a. This chemotaxis inhibitory protein of *S. aureus* (CHIPS) is a 14.1-kD protein encoded on a bacteriophage and is found in >60% of clinical isolates. CHIPS reduces the neutrophil recruitment toward C5a in a mouse peritonitis model, even though its activity is much more potent on human than on mouse cells. These findings suggest a new immune escape mechanism of *S. aureus* and put forward CHIPS as a potential new anti-inflammatory therapeutic compound.

Key words: *Staphylococcus aureus* • phagocytes • chemotaxis • C5a • formylated peptides

## Introduction

*Staphylococcus aureus* is a Gram-positive pathogen and a major cause of wound and nosocomial infections in humans causing high morbidity and mortality (1). *S. aureus* produces a wide variety of exoproteins that contribute to its ability to colonize and cause disease in the host (2). The recent increase in septic *S. aureus* diseases and in strains resistant to virtually all available antibiotics puts urgency to a better understanding of staphylococcal virulence strategies and new means to combat *S. aureus* infections (3).

An immediate host response toward bacterial infection is the migration of leukocytes from the circulation into the sites of infection. Numerous exogenously and endogenously produced leukocyte chemoattractants that initiate leukocyte migration and activation have been identified. They include both the “classical” chemoattractants, like formylated peptides, C5a, C3a, platelet-activating factor (PAF), and leukotriene B<sub>4</sub>, as well as the superfamily of chemokines that selectively induce infiltration, trafficking,

and homing of leukocytes (4, 5). The effects of all of these chemoattractants on their target cells are mediated through specific cell surface receptors, which belong to the superfamily of seven transmembrane, heterotrimeric G protein-coupled receptors (GPCRs; 6). Neutrophils bear several members of this receptor family on their surface including the receptors for formylated peptides, C5a, C3a, PAF, and leukotriene B<sub>4</sub>, as well as the chemokine receptors CXCR1 and CXCR2 that recognize IL-8 and NAP/GRO/ENA, respectively. After activation, these receptors are desensitized to repeated stimulation with the same or other agonists, which is called homologous and heterologous desensitization, respectively. Desensitization might be critical for maintaining the capacity of the cells to sense a chemoattractant gradient (7).

Although the ability of *S. aureus* to escape from human host defenses is a well-known characteristic, the underlying mechanisms have remained unclear. Early reports suggest

C.J.C. de Haas and K.E. Veldkamp contributed equally to this work.

Address correspondence to Jos A.G. van Strijp, Eijkman-Winkler Institute, Room G04-614, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, Netherlands. Phone: 31-30-2506528; Fax: 31-30-2541770; email: j.vanstrijp@azu.nl

Abbreviations used in this paper: BODIPY, boron dipyrromethane; CHIPS, chemotaxis inhibitory protein of *Staphylococcus aureus*; Eap, extracellular adherence protein; GPCR, G protein-coupled receptor; HSA, human serum albumin; MIP-1 $\alpha$ , macrophage inflammatory protein 1 $\alpha$ ; PAF, platelet-activating factor.

that some *S. aureus* strains possess an activity to delay leukocyte migration toward the site of infection. In 1967, Agarwal (8) discovered a strong strain-dependent difference in the ability to produce a purulent lesion after subcutaneous injection of staphylococci in mice. Mice challenged with "virulent" strains showed a 2-h delay in leukocyte influx and accumulation of edema fluid compared with "nonvirulent" strains. It was postulated that these virulent strains inhibit early leukocyte migration toward the site of infection (8). Hill (9) reported in 1968 that in contrast to nonvirulent strains, isolated cell wall fractions of "mouse-virulent" strains of *S. aureus* inhibited the accumulation of edema fluid at the site of injection. In 1976, Russell et al. (10) found chemotaxis-inhibiting properties for neutrophils and monocytes of several *S. aureus* strains in a modified Boyden chamber system.

These reports prompted us to look further into the proposed chemotaxis-inhibiting properties of *S. aureus*. Recently, we described an extracellular component produced by *S. aureus* that specifically impaired neutrophil chemotaxis toward fMLP, a synthetic formylated peptide, and C5a (11). In this study, we isolated and identified this substance as a new protein, chemotaxis inhibitory protein of *S. aureus* (CHIPS), which is able to specifically inhibit C5a- and fMLP-induced responses of neutrophils and monocytes. By constructing an *S. aureus* CHIPS knockout and complemented strain, we show that the activity completely matched with the presence of the CHIPS gene. CHIPS is produced by *S. aureus* in vivo and is also able to reduce the C5a-induced recruitment of neutrophils into the peritoneal cavity in a mouse model.

## Materials and Methods

**Reagents.** fMLP and C5a were obtained from Sigma-Aldrich. IL-8 and macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ) were purchased from PeproTech. Boron dipyrromethane (BODIPY)- and FITC-labeled fMLP (BODIPY-N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys), BCECF-AM, and Fluo-3-AM were obtained from Molecular Probes. S5/1 mAb (anti-CD88; C5aR) was provided by O. Götze (University Göttingen, Göttingen, Germany). SE-2 (anti-CD128w; IL-8RA) and anti-PAFR mAb were from Qbiogene. The secondary FITC-labeled goat F(ab')<sub>2</sub> anti-mouse Ig was from DakoCytomation. MMK-1 peptide (LESIFRSLL-FRVM) was synthesized by Sigma-Genosys.

**Chromatography.** Chromatographic procedures were performed with FPLC<sup>TM</sup> equipment from Amersham Biosciences at 4°C. *S. aureus* Newman or clinical isolate 1690 supernatant (11) was passed over a reactive Yellow 86, a deoxyribonucleic acid cellulose, and a Reactive Green 19 column, respectively (all from Sigma-Aldrich), coupled in tandem. The Reactive Green column was eluted with 2 M NaCl containing 1 mM phenylmethylsulfonyl fluoride. The eluate was passed over a Superdex 200 HR column (Amersham Biosciences). Fractions were screened in a flow cytometer for inhibition of binding of BODIPY-labeled formylated peptides to neutrophils as previously described (11). Active fractions were applied to a Mono Q HR anion exchange column (Amersham Biosciences) in 10 mM Tris-HCl, pH 8.0, and eluted with 0–1 M NaCl. Active fractions were analyzed on 12.5% SDS-PAGE.

**Receptor Expression on Human Neutrophils and Chemotaxis.** Human neutrophil isolation and receptor expression were performed as previously described (11). In brief, human neutrophils were incubated with increasing concentrations of CHIPS for 30 min at 37°C in RPMI containing 0.05% human serum albumin (HSA; RPMI/0.05% HSA). The samples were then incubated with 10  $\mu$ g/ml anti-C5aR, anti-IL-8RA, anti-PAFR mAb, or 1  $\mu$ M BODIPY-labeled fMLP on ice for 30 min. After washing twice, the neutrophils, incubated with mAbs, were incubated with FITC-labeled goat F(ab')<sub>2</sub> anti-mouse Ig for 30 min on ice. Samples were analyzed on a FACScan<sup>TM</sup> flow cytometer (Becton Dickinson). Chemotaxis of human neutrophils toward chemoattractants C5a and fMLP was determined using BCECF-labeled neutrophils as previously described (11). In brief, neutrophils were incubated with increasing concentrations of CHIPS for 30 min at 37°C in RPMI/0.05% HSA. The cells were then washed once and resuspended in HBSS/1% HSA (2.5  $\times$  10<sup>6</sup> c/ml). The cells (100  $\mu$ l) were added to the upper compartment of a Transwell system (3  $\mu$ m; Costar), which was placed into a 24-well plate containing 600  $\mu$ l HBSS/1% HSA, 10<sup>-7</sup> M fMLP or 10<sup>-10</sup> M C5a. After 30 min of incubation at 37°C/5% CO<sub>2</sub>, the inlays were removed and the fluorescence of the well was read in a Cytofluor<sup>II</sup> multiwell fluorometer (PerSeptive Biosystems).

**NH<sub>2</sub>-terminal Sequencing.** For NH<sub>2</sub>-terminal sequencing, the purified protein was blotted onto a polyvinylidene difluoride membrane and stained with Coomassie brilliant blue according to the manufacturer's instructions (ProBlott; Applied Biosystems). The band of interest was excised and sequenced by the Sequence Center Utrecht.

**DNA Sequencing.** Genomic DNA of *S. aureus* Newman was isolated as previously described (12). A set of degenerative primers was designed on basis of the first 32 sequenced amino acids taking into account the codon usage of staphylococci and used to directly sequence the genomic *S. aureus* Newman DNA by cycle sequencing as previously described (13). With primer 5'-GAAAAA-GAAAAAGCATATAAAGAA-3' a sequence was obtained, which was extended by primer walking. The program tblastn with the nonredundant DNA database and the microbial genomes database at <http://www.ncbi.nlm.nih.gov> was used to perform sequence similarity searches. The *chp* sequence is available from GenBank/EMBL/DDBJ under accession number AF 285146.

***chp* Knockout.** To inactivate the *chp* gene of *S. aureus* Newman, DNA fragments of 840 bp upstream and 510 bp downstream of *chp* were amplified by PCR and cloned together with the *ermB* gene from Tn551 into the temperature-sensitive shuttle plasmid pBT2 as depicted in Fig. 2 A. After cloning in *Escherichia coli* DH5 $\alpha$ , the resulting plasmid pBT $\Delta$ chp was transformed into *S. aureus* Newman to achieve integration of the *ermB* gene into the genome by homologous recombination. Mutants were enriched by cultivation at 42°C in the presence of 2.5  $\mu$ g/ml erythromycin. The proper integration of *ermB*, which is considerably larger than *chp*, was confirmed by PCR analysis. A 290-bp fragment encoding the mature portion of CHIPS was deleted. To complement the knockout strain with the *chp* gene, plasmid pRBchp was constructed by cloning a 1,293-bp PCR fragment bearing the *chp* gene together with the putative promoter region (738 bp noncoding upstream DNA) into the shuttle vector pRB473. *E. coli*- and *Staphylococcus*-specific plasmid vectors and molecular methods used have been described (13–16).

**Expression of CHIPS in *E. coli*.** The *chp* gene, except for the signal sequence, was amplified by PCR on chromosomal DNA of *S. aureus* Newman using *Pwo* DNA polymerase (Roche Diagnostics). The PCR product was cloned into the pTrcHISB vec-

tor (Invitrogen) directly downstream the enterokinase cleavage site. The vector was transformed into TOP10 *E. coli* and recombinant CHIPS was expressed and purified according to the manufacturer's instructions (Invitrogen).

**Detection of *chp* in Clinical *S. aureus* Isolates.** PCR was performed on the chromosomal DNA of 244 clinical *S. aureus* isolates using the forward primer 5'-GAAAAAGAAATTAGCAACAA-CAG-3' and the reverse primer 5'-CATAAGATGATTTA-GACTCTCC-3'.

**CHIPS Binding to Blood Cells.** To determine the binding of CHIPS to different cell types, FITC-labeled CHIPS (CHIPS-FITC) was used. FITC-labeling of CHIPS was performed as follows. 500 µg/ml CHIPS was incubated with 50 µg/ml FITC in 0.1 M sodium carbonate buffer, pH 9.6, for 1 h at 37°C. Subsequently, CHIPS-FITC was separated from unbound FITC using a G25-desalting column (Amersham Biosciences). Fractions were collected and tested for the presence of CHIPS (OD280) and FITC (OD496) in a spectrophotometer. Fractions containing CHIPS-FITC were pooled. Human neutrophils and mononuclear cells were incubated with increasing concentrations of CHIPS-FITC for 45 min on ice in the presence of PE-conjugated anti-CD14 mAb (Becton Dickinson). Samples were analyzed on a FACScan™ flow cytometer using scatters and anti-CD14 staining to distinguish the different cell populations. In separate experiments, CHIPS-FITC binding was tested on neutrophils of mice, rabbits, rats, guinea pigs, pigs, and dogs. Leukocytes were isolated from heparinized blood by either red blood cell lysis of whole blood or standard Ficoll-Histopaque isolation as for human cells. Specific binding of CHIPS-FITC was determined to neutrophils identified by their characteristic scatter pattern.

**Calcium Mobilization.** Calcium mobilization with isolated human neutrophils and monocytes (17) was performed as previously described (11). In brief, Fluo-3-loaded cells were incubated with or without 1 µg/ml CHIPS. Basal calcium levels were then measured in the FACSCalibur™ flow cytometer (Becton Dickinson),

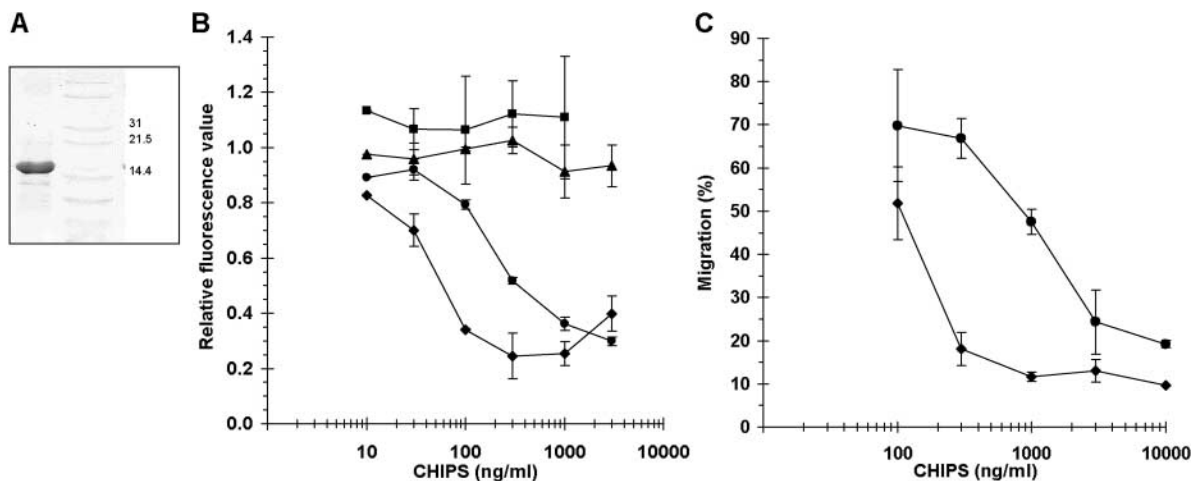
after which agonist ( $10^{-9}$  M fMLP,  $10^{-10}$  M C5a,  $10^{-10}$  M IL-8, or  $10^{-10}$  M MIP-1α) was added. 5 min after the first stimulus, the neutrophils were rechallenged with a second agonist. In separate experiments, different concentrations of CHIPS were tested on mouse and human whole blood leukocytes. Specific neutrophil response to  $10^{-10}$  M C5a was measured by electronic gating on scatters (human) and specific anti-mouse granulocyte mAb staining (R-PE-conjugated RB6-8C5; Caltag).

**CHIPS Production In Vivo.** *S. aureus* Newman or its *chp* deletion mutant were grown overnight on a blood agar plate and washed in PBS. C57Bl mice were challenged intraperitoneally with 200 µl ( $10^9$  CFU) *S. aureus* Newman or its *chp* deletion mutant. After 4 h, mice were killed, the peritoneal lavage (2.5 ml) was collected, and the amount of CHIPS was determined with a specific ELISA using a mAb as capture and a polyclonal rabbit IgG as detection antibody. Recombinant CHIPS was used as the reference standard protein. The ELISA has a limit of detection of 100 pg/ml. All animal experiments were performed in compliance with University of Utrecht guidelines and were approved by the institutional animal care and ethics committee.

**Mouse Neutrophil Influx Model.** 8–12-wk-old C57Bl mice were pretreated by intravenous injection of 100 µl PBS containing 0, 3, 10, or 30 µg CHIPS and after 15 min they were challenged intraperitoneally with 10 µg human C5a. After 3 h, mice were killed, the peritoneal lavage was collected, and the number of emigrated neutrophils was quantified and analyzed by flow cytometry by differential staining using CyChrome-conjugated anti-mouse CD45 (BD Biosciences) and R-PE-conjugated anti-mouse granulocyte (RB6-8C5).

## Results

**Isolation of CHIPS.** The chemotaxis-inhibiting component in the supernatant of the clinical strain *S. aureus* 1690 (11) was purified by ligand dye chromatography, gel



**Figure 1.** Purified CHIPS inhibits neutrophil chemotaxis toward C5a and fMLP. (A) CHIPS was purified by ligand dye chromatography and gel filtration from *S. aureus* culture supernatant. Active fractions were pooled and concentrated. SDS-PAGE revealed a band of ~15 kD. The right lane represents a marker. (B) CHIPS affects C5aR and FPR expression on neutrophils in a dose-dependent fashion. Binding of BODIPY-formylated peptide (◆) and anti-C5aR mAb (●) to their receptors on neutrophils is inhibited after 30 min of incubation with different concentrations of CHIPS, whereas binding of anti-PAFR (■) and anti-IL-8R (▲) mAb is not inhibited. Data are expressed as fluorescence values compared with neutrophils without CHIPS and are mean ± SEM of three separate experiments. (C) CHIPS inhibits chemotaxis of neutrophils toward  $10^{-7}$  M fMLP (◆) and  $10^{-10}$  M C5a (●) after incubation of neutrophils with different concentrations of CHIPS. Data are expressed as the percentage of migrated neutrophils added to the upper compartment of the transwell chemotaxis chamber and are mean ± SEM of one representative experiment ( $n = 2-4$ ). Migration of untreated neutrophils was 79% for C5a and 85% for fMLP.

permeation, and anion exchange chromatography. To detect the samples containing the chemotaxis-inhibiting activity, all samples were screened by flow cytometry for the inhibition of binding of fluorescent-labeled formylated peptides to neutrophils. The final preparation revealed a nearly homogeneous protein band of ~15 kD on SDS-PAGE (Fig. 1 A). The purified protein exhibited the same activity as the supernatant. It inhibited binding of formylated peptides and anti-C5aR antibodies to neutrophils (Fig. 1 B) as well as neutrophil chemotaxis toward fMLP and C5a (Fig. 1 C). Neither expression of the receptors for IL-8 and PAF were affected nor their chemotactic activity. The protein itself showed no direct chemotactic activity (not shown). NH<sub>2</sub>-terminal sequencing identified the first 32 amino acids, which showed no major homology with any protein in databases. We named this new protein chemotaxis inhibitory protein of *S. aureus* (CHIPS).

**Identification of the CHIPS Gene.** To identify the CHIPS gene, we performed direct sequencing on genomic DNA of *S. aureus* Newman, which is also a CHIPS-producing strain. This yielded a DNA sequence encoding a protein of 149 amino acids: MKKKLATTVLALSFLTAGISTHHHSAKA-**FTFEPFPPTNEEIESNKKMLEKEKAYKESFKNSGLP-TTLGKLDERLRNYLKKKGTKNSAQFEKMKVILTEN-KGYYTVYLNTPLAEDRKNVELLQKMYKTYFFKK-GESKSSYVINGPGKTNEYAY**. The NH<sub>2</sub>-terminal 28 amino acids constituted a signal peptide containing a signal peptidase 1 cleavage site (underlined) followed by amino acids that perfectly matched the NH<sub>2</sub>-terminal CHIPS sequence (bold). The mature protein had a mass of 14.1 kD. The gene, named *chp*, was preceded by a Shine Dalgarno sequence and followed by three stop codons. In strain Newman *chp* was found to be located on the 3' end of a bacteriophage. This bacteriophage is incorporated in the gene for β-toxin (*hlyB*) and

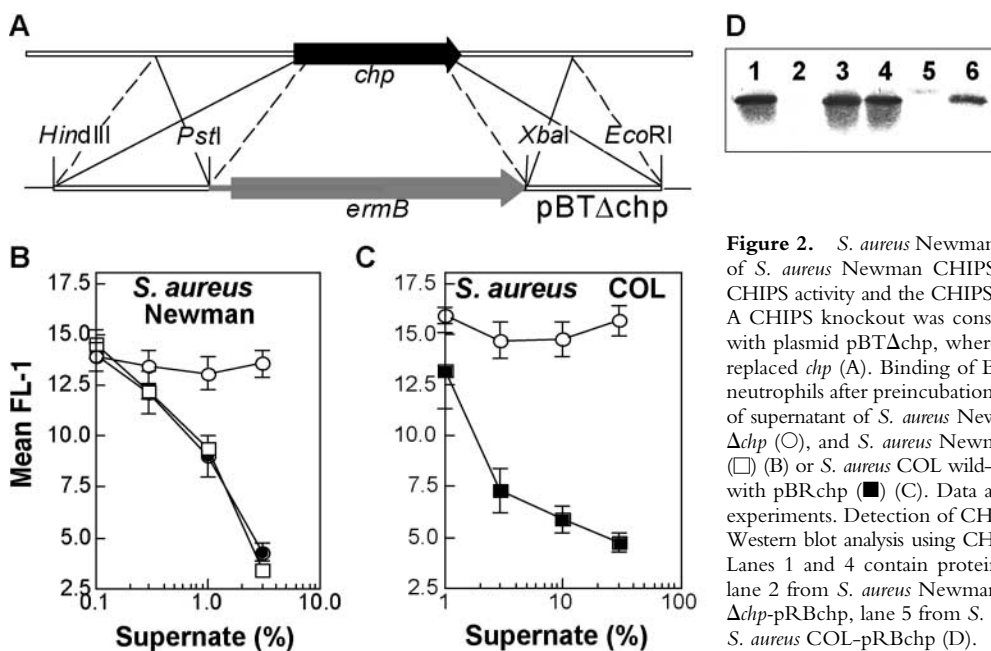
its 3' end carries, beside *chp*, the genes for staphylokinase (*sak*) and staphylococcal enterotoxin A (*sea*).

**CHIPS Knockout *S. aureus* Strain Lacks Chemotaxis-inhibiting Activity.** To confirm that *chp* was responsible for the CHIPS activity in the *S. aureus* supernatant, a CHIPS knockout strain was constructed by replacement of *chp* with an erythromycin resistance cassette (Fig. 2 A). This resulted in total loss of CHIPS activity and absence of the protein in the culture supernatant (Fig. 2, B and D). When the knockout strain was complemented with plasmid pRB-*chp*, containing *chp* with its putative promoter region, CHIPS activity was restored. When *S. aureus* COL (*chp* negative by PCR) was transformed with pRB*chp*, it showed CHIPS activity and produced the protein (Fig. 2, C and D). These data show that CHIPS activity correlates with the presence of *chp*. Additionally, recombinant CHIPS produced by *E. coli* had exactly the same activity as native *S. aureus* CHIPS (unpublished data).

**Prevalence in Clinical *S. aureus* Isolates.** To investigate the prevalence of *chp* in clinical *S. aureus* isolates, we screened 204 methicillin-sensitive isolates (102 from blood stream infections and 102 from healthy nasal carriers) and 40 methicillin-resistant *S. aureus* strains by PCR. Of all strains, 62% were positive for *chp* and the presence of *chp* neither correlated with the clinical source of the strains nor differed between methicillin-sensitive and methicillin-resistant isolates.

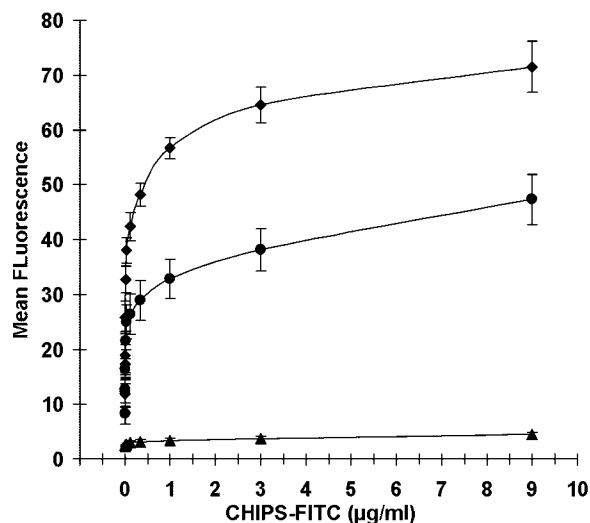
**CHIPS Binds to Human Neutrophils and Monocytes.** To examine the binding capacity of CHIPS to different leukocytes, FITC-labeled CHIPS was used. CHIPS specifically bound to human neutrophils and monocytes and not to lymphocytes (Fig. 3), which indicates its specificity for those cells that pose the greatest threat for invading staphylococci.

**CHIPS Specifically Inhibits the fMLP- and C5a-induced Calcium Mobilization.** The hallmark for the GPCR is a rapid and transient increase in free intracellular calcium



**Figure 2.** *S. aureus* Newman CHIPS knockout strain. Construction of *S. aureus* Newman CHIPS knockout results in the total loss of CHIPS activity and the CHIPS protein band in the culture supernatant. A CHIPS knockout was constructed by homologous recombination with plasmid pBTΔ*chp*, whereby an erythromycin resistance cassette replaced *chp* (A). Binding of BODIPY-labeled formylated peptide to neutrophils after preincubation with different concentrations (vol/vol) of supernatant of *S. aureus* Newman wild-type (●), *S. aureus* Newman Δ*chp* (○), and *S. aureus* Newman Δ*chp* complemented with pRB*chp* (□) (B) or *S. aureus* COL wild-type (○) and *S. aureus* COL transformed with pRB*chp* (■) (C). Data are mean ± SEM of three independent experiments. Detection of CHIPS in *S. aureus* culture supernatants by Western blot analysis using CHIPS-specific polyclonal rabbit antiserum. Lanes 1 and 4 contain proteins from *S. aureus* Newman wild-type, lane 2 from *S. aureus* Newman Δ*chp*, lane 3 from *S. aureus* Newman Δ*chp*-pRB*chp*, lane 5 from *S. aureus* COL wild-type, and lane 6 from *S. aureus* COL-pRB*chp* (D).



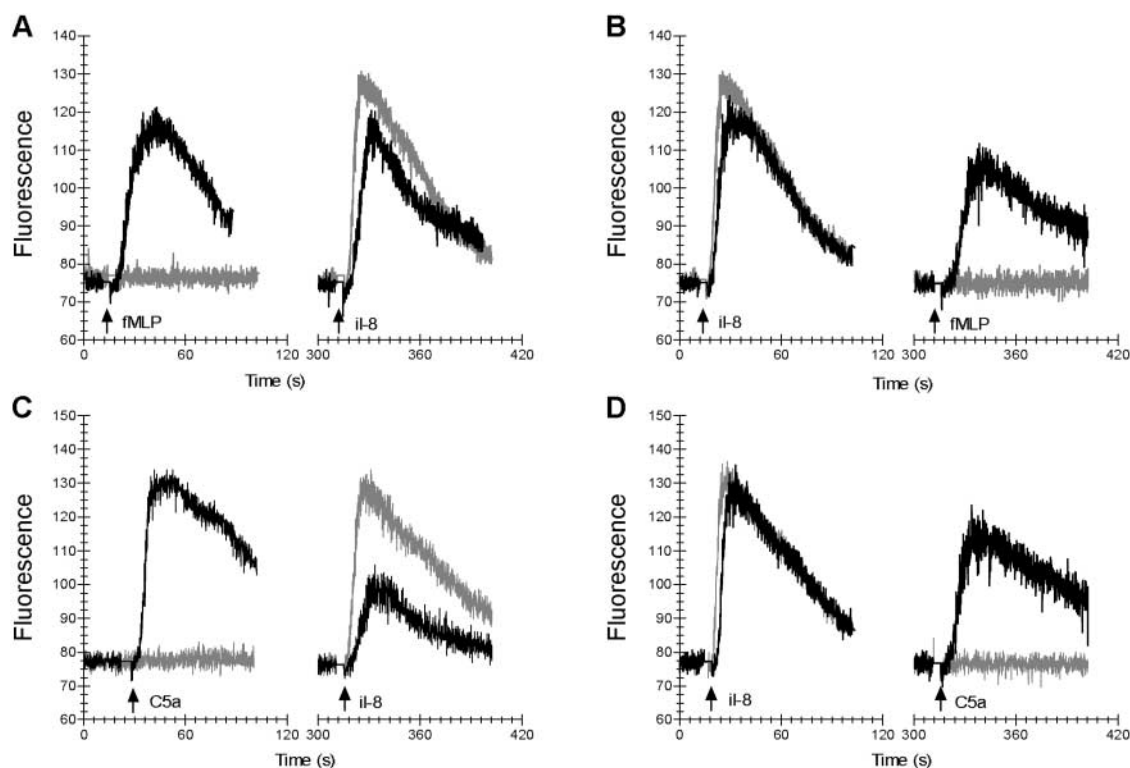


**Figure 3.** CHIPS binds to phagocytes. Dose-dependent binding of CHIPS-FITC to neutrophils (◆), monocytes (●), and lymphocytes (▲). Cells were identified based on scatter parameters and anti-CD14 staining. Data are mean ± SEM of five independent experiments.

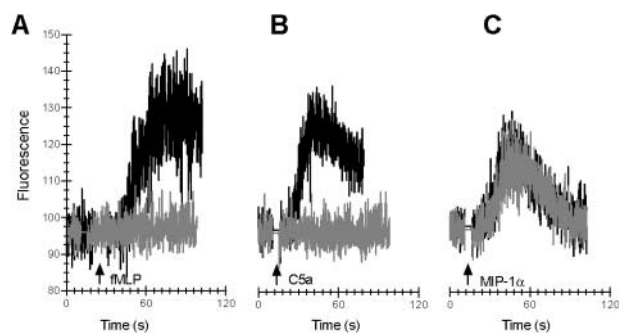
upon ligand binding (6, 18). The inhibiting effect of CHIPS on the fMLP- and C5a-induced calcium mobilization in human neutrophils was not due to desensitization. CHIPS specifically blocked the calcium mobilization of

neutrophils induced by fMLP or C5a, but not IL-8 (Fig. 4). CHIPS-treated cells, triggered with fMLP or C5a, responded with a normal calcium response to a rechallenge with IL-8 (Fig. 4, A and C). Vice versa, CHIPS-treated cells were normally activated by IL-8, but failed to mount a calcium mobilization upon rechallenge with fMLP or C5a (Fig. 4, B and D). Buffer-treated cells were unaffected in their primary response to fMLP, C5a, and IL-8, whereas their secondary response to a heterologous stimulus was lower due to desensitization. The full secondary response to IL-8 of CHIPS-treated cells, preexposed to fMLP or C5a, can be explained by the efficient FPR and C5aR receptor blockade by CHIPS. CHIPS itself did not induce a calcium response. CHIPS also inhibited the fMLP- and C5a-induced calcium mobilization in monocytes, whereas the response to MIP-1α was not affected (Fig. 5). In addition, CHIPS did not inhibit human neutrophils triggered with an FPRL1-specific agonist, the synthetic peptide MMK-1 derived from a random peptide library that does not activate FPR (unpublished data and 19). We conclude that CHIPS affects specifically two members of the GPCR family and is not toxic for cells because the cellular response to MMK-1, IL-8, and MIP-1α remained intact.

*CHIPS Is Human Specific.* As *chp* is located on a bacteriophage together with the two human-specific virulence genes, *sak* and *sea*, we tested the species specificity of



**Figure 4.** CHIPS specifically inhibits fMLP- and C5a-induced calcium mobilization in neutrophils. The effect of CHIPS on intracellular-free calcium release induced by  $10^{-9}$  M fMLP,  $10^{-10}$  M C5a, and  $10^{-10}$  M IL-8 in neutrophils was determined using Fluo-3-labeled cells and flow cytometry. After preincubating neutrophils with 1 µg/ml CHIPS (gray lines) or buffer (black lines), the basal fluorescence level was measured for each sample before fMLP (A), C5a (C), or IL-8 (B and D) was added (indicated by first arrow). To show receptor specificity, cells were rechallenged with a second stimulus of fMLP (B), C5a (D), or IL-8 (A and C) after 5 min (indicated by second arrow).



**Figure 5.** CHIPS specifically inhibits fMLP- and C5a-induced calcium mobilization in monocytes. The effect of CHIPS on intracellular-free calcium release induced by  $10^{-9}$  M fMLP,  $10^{-10}$  M C5a, and  $10^{-10}$  M MIP-1 $\alpha$  in monocytes was determined using Fluo-3-labeled cells and flow cytometry. After preincubating monocytes with 1  $\mu$ g/ml CHIPS (gray lines) or buffer (black lines), the basal fluorescence level was measured for each sample before fMLP (A), C5a (B), or MIP-1 $\alpha$  (C) was added (indicated by arrow).

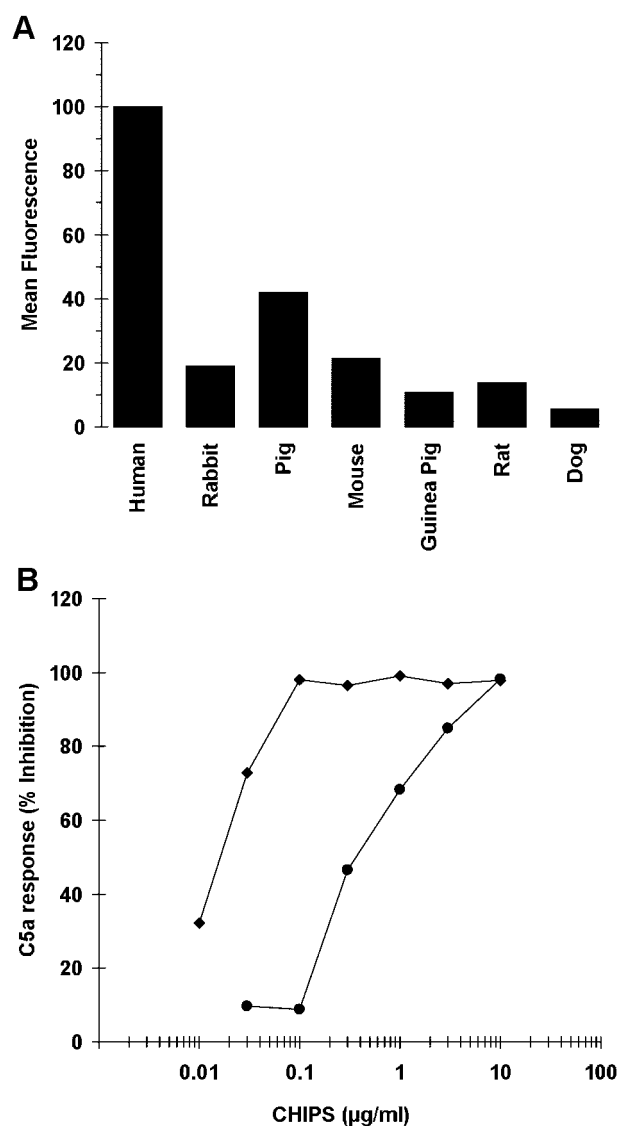
CHIPS. Binding of FITC-labeled CHIPS to isolated neutrophils of different animal species revealed a low binding to neutrophils of all species tested when compared with human neutrophils (Fig. 6 A). Furthermore, the efficacy of CHIPS to inhibit the C5a-induced calcium mobilization was evaluated with human and mouse neutrophils. CHIPS showed a 30-fold reduced capacity to inhibit the C5a-induced calcium mobilization in mouse neutrophils as compared with human neutrophils (Fig. 6 B). These results suggest that CHIPS is human specific.

**CHIPS Is Produced In Vivo.** CHIPS is secreted by *S. aureus* in vitro in sufficient amounts to inhibit neutrophil activation. However, to demonstrate a role for CHIPS in the virulence of *S. aureus*, we examined whether *S. aureus* produces detectable amounts of CHIPS in vivo. Therefore, mice were injected intraperitoneally with  $10^9$  CFU *S. aureus* Newman or the CHIPS knockout strain. After 4 h,  $8.18 \pm 2.89$  ng CHIPS was recovered from the peritoneal lavage of mice injected with *S. aureus* Newman ( $n = 6$ ), whereas no CHIPS was found in knockout strain-injected mice ( $n = 2$ ). This shows that in a localized infection, *S. aureus* actively secretes only minimal amounts of CHIPS in the surroundings.

**CHIPS Inhibits the Mouse Neutrophil Influx In Vivo.** To investigate the potency of CHIPS to specifically inhibit the neutrophil migration in vivo, mice were intravenously treated with CHIPS and subsequently, after 15 min, intraperitoneally challenged with human C5a. At least 10  $\mu$ g of CHIPS per mouse was needed to give a 65% inhibition of neutrophil influx (Fig. 7). These results indicate a potential use of CHIPS as an antiinflammatory drug in diseases where influx of neutrophils is more detrimental than beneficial for the host.

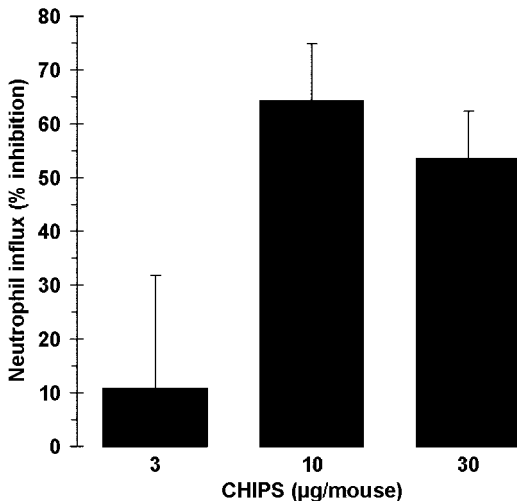
## Discussion

When *S. aureus* invades the human host, complement is rapidly activated, resulting in opsonization of the bacteria and the generation of large amounts of C5a (20–22). To-



**Figure 6.** CHIPS binding to neutrophils of different species. (A) Leucocytes of different species were incubated with 1  $\mu$ g/ml CHIPS-FITC and analyzed by flow cytometry based on scatter parameters to identify neutrophils. Data shown are from one representative experiment. (B) The effect of different concentrations CHIPS on the C5a-induced calcium mobilization in human ( $\blacklozenge$ ) and mouse ( $\bullet$ ) neutrophils. Data are expressed as percentage inhibition of the response of control cells to  $10^{-10}$  M C5a ( $n = 2$ ). The response was defined by the difference in mean fluorescence (MFL) of basal level and maximal level after challenge ( $\Delta$ MFL = 84.3 for human and 22.9 for mouse).

gether with formylated peptides, side products of bacterial translation, generation of C5a constitutes the first trigger of the innate immune system (23). These early signs of bacterial invasion are recognized by the innate immune system through two related receptors on neutrophils, the C5aR and FPR. In vitro, in vivo, and possibly during *S. aureus* infection, recognition by these two receptors is counteracted by CHIPS as it inhibits specifically the C5a- and fMLP-induced responses in neutrophils and monocytes. Moreover, CHIPS inhibits the neutrophil recruitment toward C5a in a



**Figure 7.** CHIPS inhibits the C5a-induced neutrophil migration in vivo. Mice were pretreated by intravenous injection of different concentrations of CHIPS and after 15 min were challenged intraperitoneally with human C5a. After 3 h, the percentage of neutrophils in the peritoneal lavage was analyzed. Data are expressed as percentage inhibition compared with buffer-treated mice challenged with C5a after correction for control nontreated mice. Data are mean  $\pm$  SEM of two to three separate experiments ( $n = 2$  mice per treatment).

mouse peritonitis model. Because CHIPS did not inhibit receptors for secondary chemoattractants, such as IL-8, we hypothesize that CHIPS plays a major role early in bacterial invasion. Once staphylococci have multiplied abundantly, the overwhelming presence of other chemokines will overcome CHIPS inhibitory effects causing massive influx of neutrophils. This results in the typical hallmark of a staphylococcal infection, a purulent lesion (1). As yet, it is not known how CHIPS expression is regulated, but we would predict from its ligand specificity that CHIPS expression is most abundant at the onset of infection.

As CHIPS is an actively excreted protein of *S. aureus*, the *S. aureus*-induced chemotaxis-inhibiting findings of Agarwal (8) might be attributed to the in vivo production of CHIPS by the virulent *S. aureus* strains. The observation of a 2-h delay in leukocyte migration toward the infected site would correlate with our hypothesis of an early effect of CHIPS inhibiting the leukocyte recruitment toward the two first chemoattractants produced, formylated peptides and C5a. Recently, extracellular adherence protein (Eap) of *S. aureus* was reported to inhibit leukocyte chemotaxis by its interaction with intercellular adhesion molecule 1 (24). Like CHIPS, Eap was produced by *S. aureus* Newman in vivo, which suggests that this strain has at least two different mechanisms to avoid leukocyte recruitment (25).

Given its potent action on human phagocytes, CHIPS may represent an important virulence factor. This seems to be in agreement with the abundance of *chp* (62%) in *S. aureus* isolates. The presence of *chp* did not correlate with the development of bacteraemia. There are two different explanations for this. We hypothesize that CHIPS is mainly important in the initial interaction with the host and mainly

contributes to effective colonization or early stages of infection. In addition, there might be some redundancy in virulence factors, such as Eap, which avoid the innate immune system. Once the bacterium harbors more than a threshold number of these factors, the bacterium becomes pathogenic. This research includes CHIPS as one of these factors.

The human specificity of CHIPS as shown by a 30-fold difference in activity toward human cells as compared with mouse cells, in combination with its low in vivo expression, hampers testing of CHIPS in a mouse infection model or other animal models. Apart from *chp*, the other adjacent genes *sak* and *sea* have also been reported to be highly human specific. The response of mouse T cells to the superantigen staphylococcal enterotoxin A requires 1,000-fold higher concentrations compared with human T cells (26). In addition, the ability of bacterial plasminogen activators to cleave different animal plasminogen is restricted. Staphylokinase could only activate plasminogen of humans, dogs, and baboons (27). The exact role of CHIPS in *S. aureus* virulence will be a subject for future studies. As an in vivo infection model was not possible due to the human specificity of CHIPS, we included a mouse peritonitis model with purified CHIPS. As we could simply overdose CHIPS in this model, we were able to show a proof of principle for the in vivo anti-chemotactic action of CHIPS.

CHIPS fulfils a unique function, affecting two related receptors with strikingly different ligand specificities. Both the C5aR and FPR belong to the superfamily of seven transmembrane, heterotrimeric GPCRs (28, 29). All members of this family have a comparable architecture and show a 20–30% amino acid homology, mainly in the transmembrane regions, whereas the ligands are highly diverse and receptor specific. Small molecules, such as fMLP, bind to their GPCR in the interhelical region, whereas larger molecules, such as C5a, use a two-site motif for binding their GPCR (30, 31).

How CHIPS interferes with both fMLP and C5a responses is still speculative. It is unlikely that CHIPS affects common downstream signaling events related to the GPCR as was shown for pertussis toxin (32), because the MMK-1, IL-8, and MIP-1 $\alpha$  responses were unaffected. Binding of CHIPS-FITC was restricted to phagocytes, in which neutrophils showed a twofold higher binding than monocytes. This coincides with the expression of the C5aR and FPR on the respective cell types (33–36) and could therefore implicate direct binding to these chemokine receptors. The fact that CHIPS binds to phagocytes implies that CHIPS probably is not a protease-like protein cleaving (a part of) the receptor, as was described for other receptors (37, 38). Alternative explanations, in which CHIPS could act via a separate CHIPS receptor or interferes with proper surface exposure of C5aR and FPR by mimicking conserved receptor transmembrane regions (39), do not provide a plausible explanation for its narrow specificity for C5aR and FPR. The exact mechanism of CHIPS inhibiting fMLP- and C5a-induced responses will be further investigated.

We present data that implicate a new virulence strategy of *S. aureus* and thereby a new potential target in the treatment of *S. aureus* infection. In addition, the potent capacity of CHIPS to inhibit neutrophil chemotaxis, in vitro and in vivo, makes this new protein a promising candidate anti-inflammatory drug for those diseases in which C5a-induced damage by neutrophils plays a pivotal role (40–44).

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